Effects of heparin and lisofylline on pulmonary function after smoke inhalation injury in an ovine model*

Osamu Tasaki, MD; David W. Mozingo, MD; Michael A. Dubick, PhD; Cleon W. Goodwin, MD; Loudon D. Yantis, DVM; Basil A. Pruitt Jr, MD

Objective: **This study evaluates the effects of heparin alone and in combination with lisofylline, 1-(5-R-hydroxyhexyl)3,7-dimethylxanthine, on severe smoke injury.**

Design: **Prospective animal study with concurrent controls.**

Setting: **An animal laboratory.**

Subjects: **Eighteen 1-yr-old female sheep, weighing 24–32 kg.**

Interventions: **After smoke exposure and tracheostomy, animals were divided into three groups. Group S (n = 6) received nebulized saline through an endotracheal tube every 4 hrs for 48 hrs. Group H (n = 6) received 10,000 units of nebulized heparin every 4 hrs. Group LH (n = 6) was treated with nebulized heparin and intravenous infusion of lisofylline (10 mg·kg^{-1}·hr^{-1}) for 48 hrs after a bolus injection (20 mg/kg). Animals initially breathed room air spontaneously. If Pa_{O_2} was <50 torr and Pa_{CO_2} >60 torr, animals were mechanically ventilated. Sheep were killed 48 hrs postinjury.**

Measurements and Main Results: **Blood gases were measured serially. At 48 hrs, ventilation perfusion distribution mismatching was analyzed by using the multiple inert gas elimination technique. Lung malondialdehyde was determined. The postinjury increase in alveolar-arterial oxygen tension gradient (LH, 36.7 ± 3.5 vs. S, 89.0 ± 24.6 torr at 48 hrs) was significantly attenuated in those animals receiving LH. The percentage of pulmonary shunt, Qs/Qt (LH, 20.8 ± 4.9 vs. S, 36.6 ± 4.6%), and the percentage of animals that required ventilation (LH, 0 vs. S, 67%) were significantly reduced in LH. Multiple inert gas elimination technique study showed that the true shunt fraction was decreased in LH. Lung malondialdehyde was significantly less in LH (LH, 0.33 ± 0.06 vs. S, 0.56 ± 0.09 nmol/mg protein). There was no significant difference in any of these variables between H and S.**

Conclusion: **Treatment with heparin alone did not attenuate pulmonary dysfunction after severe smoke injury. Combined treatment with nebulized heparin and systemic lisofylline had beneficial effects on pulmonary function in association with a decrease in blood flow to poorly ventilated areas and less lipid peroxidation. (Crit Care Med 2002; 30:637–643)**

KEY WORDS: smoke inhalation injury; lisofylline; ovine; heparin; leukocyte; malondialdehyde; multiple inert gas elimination technique; cast formation; free radical; pulmonary dysfunction

Smoke inhalation injury remains a significant comorbid factor of burned patients. Patients with both injuries are more hemodynamically unstable than patients with a comparable burn injury alone, and concomitant inhalation injury results in higher mortality rates than would be predicted on the basis of age and burn size (1–3). Therefore, it is important to develop a treatment strategy to improve the survival of burned patients who also have inhalation injury.

Treatment with heparin has been shown to exert beneficial effects in patients with smoke inhalation injury. Cox et al. (4) reported that intravenous administration of heparin improved oxygenation and minimized barotrauma after severe smoke inhalation injury in an ovine model by decreasing tracheobronchial cast formation and pulmonary edema. On the other hand, Schenarts et al. (5) reported that heparin did not attenuate the acute lung injury associated with smoke inhalation in another ovine model. In short, the beneficial effects of heparin remain unconfirmed.

We previously reported that pentoxifylline decreased airway damage in a model of mild smoke inhalation injury (6). Lisofylline, 1-(5-R-hydroxyhexyl)3,7-dimethylxanthine(L), is converted to pentoxifylline in the liver (7) and has been reported to inhibit production of inflammatory mediators (8), down-regulate leukocyte activation (9), and suppress the formation of Th1 lymphocytes (10), which are thought to play important roles in the inflammatory process after smoke inhalation injury. In all, lisofylline is reportedly about 800-fold more active as an inhibitor of inflammatory mediators than pentoxifylline. In addition, lisofylline has been shown to be effective against ischemia/reperfusion injury (11).

Based on these studies, we postulated that lisofylline in combination with heparin would be effective in treating smoke inhalation injury. Therefore, the present study evaluated the effects of heparin alone and in combination with lisofylline on the pulmonary dysfunction associated with severe smoke inhalation injury.

MATERIALS AND METHODS

Animals and Instrumentation

Eighteen female sheep weighing 24–32 kg and devoid of antibodies to Q fever were used

*See also p. 718.

From U.S. Army Institute of Surgical Research (OT, MAD, CWG), San Antonio, TX; the Department of Surgery (DWM), University of Florida, Gainesville, FL; and the Health Science Center at San Antonio (BAP), University of Texas.

Supported, in part, by the U.S. Army Institute of Surgical Research.

The opinions and assertions contained herein are the private views of the authors and are not to be construed as official, nor do they reflect the views of the Department of the Army or the Department of Defense.

Address requests for reprints to: Library Branch, US Army Institute of Surgical Research, 3400 Rawley E. Chambers Avenue, Fort Sam Houston, San Antonio, TX 78234-6315.

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01 MAR 2002	**N/A**	**-**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Effects of heparin and lisofylline on pulmonary function following smoke inhalation injury in an ovine model.	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
Tasaki O., Mozingo D. W., Dubick M. A., Goodwin C. W., Yantis L. D., Pruitt B. A.,	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)	8. PERFORMING ORGANIZATION REPORT NUMBER
United States Army Institute of Surgical Research, JBSA Fort Sam Houston, TX 78234	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**SAR**	**7**	

in this study. The animals were housed in large cages, treated for parasites (Ivermectin, Rahway, NJ, 0.2 mg/kg intramuscularly), and fed commercial chow and water *ad libitum*. On the day before smoke exposure, all animals were instrumented with femoral artery and vein catheters while anesthetized with sodium pentobarbital (25 mg/kg intravenously). One radiopaque sheath introducer, through which a pulmonary artery flotation catheter was placed, was inserted into an external jugular vein. The animals then were awakened, extubated, and returned to their cages. This study was approved by our Institutional Animal Care and Use Committee. The care of all animals was in accordance with the guidelines set forth by the Animal Welfare Act and other federal statutes and regulations relating to animals and studies involving animals and with the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health Publication 86-23).

Smoke Exposure and Tracheostomy

Twenty-four hours after instrumentation, the animals were reanesthetized, were intubated with a 7.5-mm orotracheal tube, and received an inhalation injury induced by exposure to smoke as described previously (12). Briefly, sustained smoke was generated by thermolysis of pine woodchips (100 g) in a crucible furnace at a constant temperature of 752°F (400°C) and air flow of 6.0 L/min. The smoke was delivered into a 20-L reservoir, where it cooled to room temperature and was mixed with a 2.0-L/min flow of 100% oxygen. Animals received 15 exposure units of this mixture by using a hand-operated piston; one exposure unit consisted of five breaths (tidal volume, 30 mL/kg, with a breath-hold of 6 secs) with a 5-sec rest between exposure units. Immediately after smoke exposure, all animals were ventilated with 100% oxygen and received tracheostomy under anesthesia with isoflurane. After tracheostomy, the animals were housed in individual cages in a climate-controlled facility and were observed for 48 hrs while breathing room air spontaneously. In a preliminary study with 12 sheep, comparing lisofylline treatment to saline treatment, after smoke exposure animals did not receive tracheostomies.

Protocol

All animals received 5% dextrose in lactated Ringer's solution at a rate of 2 $mL \cdot kg^{-1} \cdot hr^{-1}$ for 48 hrs. A catheter was inserted into the bladder, and urine output was monitored every 4 hrs. Water balance was calculated by subtracting urine output from the volume infused. The infusion rate was changed if necessary to maintain an output of $>0.5\ mL \cdot kg^{-1} \cdot hr^{-1}$. The smoke-exposed animals then were divided into three groups. Group S (n = 6) received nebulized saline through an endotracheal tube 30 mins after smoke exposure and every 4 hrs thereafter for 48 hrs. Group H (n = 6) received 10,000 units of nebulized heparin according to the same protocol as group S. Group LH (n = 6) was treated with nebulized heparin and intravenous infusion of lisofylline (10 $mg \cdot kg^{-1} \cdot hr^{-1}$; Cell Therapeutics Inc., Seattle, WA) for 48 hrs after a bolus injection (20 mg/kg) at 30 mins after smoke exposure. This same dosage of lisofylline was used in our preliminary studies.

Measurements

Blood Gas. Blood gases were measured serially according to standard techniques by using an IL 1303 pH/blood gas analyzer and an IL 482 CO-oximeter (Instrumentation Laboratories, Lexington, MA). The samples for blood gas determination were taken while the animals were breathing room air unless they were on mechanical ventilation. In our model of smoke inhalation injury, Pa_{O_2} decreased first but was maintained for a while by spontaneous hyperventilation. As the pulmonary dysfunction progressed, Pa_{CO_2} increased and the animals developed dyspnea. Therefore, the decision to institute respiratory support (oxygen supplementation and mechanical ventilation) was based on clinical variables. If Pa_{O_2} decreased below 50 torr but Pa_{CO_2} was maintained <60 torr, animals received oxygen supplementation (100% oxygen at 5 L/min mixed with room air). Animals were off oxygen supplementation for at least 15 mins before blood sampling. If Pa_{CO_2} increased above 60 torr, animals were mechanically ventilated under anesthesia and the $F_{I_{O_2}}$ was adjusted. Ventilator settings were adjusted according to the protocol described by Cox et al. (4). Initial ventilator settings were a tidal volume of 15 mL/kg, a respiratory rate of 12 breaths/min, a positive end expiratory pressure of 5 cm H_2O, and an $F_{I_{O_2}}$ of 0.3. The respiratory rate was adjusted to maintain Pa_{CO_2} <60 torr. The $F_{I_{O_2}}$ was adjusted to maintain the Pa_{O_2} at or just above 60 torr. If >0.5 $F_{I_{O_2}}$ was required, then positive end expiratory pressure was increased in 2.5 cm H_2O increments up to 10 cm H_2O to maintain the Pa_{O_2} at or just above 60 torr.

Alveolar-arterial oxygen tension gradient, or $P(A\text{-}a)_{O_2}$, was calculated by using the following equation: $P(A\text{-}a)_{O_2} = PA_{O_2} - Pa_{O_2}$. Alveolar oxygen tension (PA_{O_2}) was calculated according to the following equation: $PA_{O_2} = F_{I_{O_2}} \times BP - Pa_{CO_2} \times ([F_{I_{O_2}}] + [1 - F_{I_{O_2}}]/R)$, where BP = barometric pressure − vapor pressure; vapor pressure = 0.4 + antilog(0.024T + 0.7659); T = absolute temperature in °C; and R (respiratory quotient) = 0.8. Blood samples were also taken for the measurement of total protein, creatinine, and glucose by using standard clinical chemistry procedures on a Hitachi 911 Clinical Chemistry Analyzer (Boehringer Mannheim, Indianapolis, IN).

Ventilation Perfusion Distribution (V_A/Q). V_A/Q mismatching was analyzed at 48 hrs by using the multiple inert gas elimination technique (MIGET) under mechanical ventilation. The animals were anesthetized with sodium pentobarbital (25 mg/kg intravenously) and paralyzed with pancuronium bromide (0.03–0.04 mg/kg, Pavulon; Organon Pharmaceuticals, West Orange NJ). During mechanical ventilation, the tidal volume was set at 15 mL/kg, the respiratory rate at 12 breaths/min, and positive end expiratory pressure at 5 cm H_2O. $F_{I_{O_2}}$ was adjusted according to the Pa_{O_2} as described previously. A lactated Ringer's solution containing six inert gases (sulfur hexafluoride, ethane, cyclopropane, halothane, diethyl ether, and acetone) was infused at a rate of 0.1 $mL \cdot kg^{-1} \cdot min^{-1}$. After 40 mins, arterial and mixed-venous blood samples (10 mL each) were simultaneously drawn anaerobically into preweighed, heparinized syringes. Mixed-expired gas was collected from a temperature-controlled (104°F [40°C]) copper coil (outer diameter, 3.5 cm; length, 550 cm) about 1 min after blood sampling, compensating for the delay of the mixing chamber. Blood and expired gas samples were immediately analyzed by gas chromatography. V_A/Q on a 50-compartment scale was calculated based on retention ratios by using a special computer program (13).

Lung Malondialdehyde (MDA), Myeloperoxidase (MPO) Activity, and Wet to Dry Weight Ratio (W/D). After the MIGET study, all animals were killed. The right lung was taken to determine W/D and to measure MDA and MPO activity. W/D was determined by a previously described method (14). Briefly, the lung was homogenized with an identical weight of distilled water. Some of the homogenized lung was stored at −80°C until MDA concentrations were measured. Samples of the homogenate and blood were weighed and dried at 80°C for 48 hrs. Dry weights were measured, and the W/D of the homogenate and blood was calculated. A sample of the homogenate was centrifuged at 12,500 rpm for 1 hr, and blood samples were diluted with the same volume of distilled water. To determine the hemoglobin concentrations in the homogenate and blood, 20 μL of the homogenate supernatant or the diluted blood was added to 2.5 mL of Drabkin's solution. The absorbance of both solutions was measured spectrophotometrically at 540 nm. Then, the weight of the blood in the wet lung was calculated. From these data, blood-free W/D was determined (14).

MDA concentrations in lung homogenates were determined in the butanol phase by the spectrophotometric assay of Naito et al. (15) by using 1,1,3,3 tetraethoxypropane as standard. MPO activity was determined by a modification of the method of Trush et al. (16). Briefly, lung samples were homogenized in 50 mM potassium phosphate buffer, pH 6.0, con-

taining 0.5% hexadecyltrimethylammonium bromide. The samples then underwent three freeze-thaw cycles and sonification, followed by incubation in a 60°C water bath for 2 hrs to extract MPO and eliminate interfering substances. Samples then were centrifuged at 10,000 × *g* for 30 mins at 4°C. MPO activity was determined in the resultant supernatant by using o-dianisidine as substrate. Protein concentration of the lung homogenates was determined by using a commercial kit (BioRad Laboratories, Richmond, CA).

Histology

The light microscopy histologic grading of the tracheobronchial and lung parenchymal injury of each animal was performed by using the previously described criteria (17). Briefly, the tracheobronchoepithelial damage score was as follows: 0, normal; 1, some loss of cilia, loss of apical epithelium; 2, marked attenuation of epithelium, single layer of epithelium; 3, <50% ulceration of epithelium; 4, >50% ulceration of epithelium. Assessment of lung parenchymal damage was limited to the left apical lobe because bronchoalveolar lavage was done in the other lobes. The lung parenchymal damage score was as follows: 0, normal; 1, minimal to mildly thickened alveolar septa, a few inflammatory cells or small, single focus on inflammatory cells; 2, multifocal areas with increased inflammatory cells in alveolar septa and in alveoli; 3, diffuse inflammation and/or edema that affects less than half of the section; 4, diffuse inflammation and/or edema that affects more than half of the section.

Statistical Analysis

Statistical analysis was performed by using repeated-measures analysis of variance with *post hoc* Scheffé's test for comparison between groups. An unpaired Student's *t*-test was used for nonrepeated measures. Fisher's exact test was used to compare the incidence of mechanical ventilatory support in each group. The Mann-Whitney U test was used for histologic evaluation. Data are shown as mean ± SEM. Significance was assigned at $p < .05$.

RESULTS

Data from our preliminary study with lisofylline alone are presented separately because these animals did not receive tracheostomies or receive nebulized saline as a control. In addition, not all variables presented in the main study were measured in the preliminary study.

Arterial carboxyhemoglobin concentrations immediately after smoke exposure were 96.5 ± 1% in group S, 95.9 ± 0.8% in group H, and 95.5 ± 0.9% in group LH. This suggested a very severe smoke injury that was similar in all groups. All animals survived the 48-hr study period. However, five of six animals in group S, two of six in group H, and one of six in group LH required oxygen supplementation attributable to hypoxemia. In addition, some animals required mechanical ventilation because of hypoxemia and hypercapnia. No animals required mechanical ventilation during the first 24 hrs after injury (Table 1). In the smoke-only group, one animal required ventilation by 36 hrs and four animals by 48 hrs, indicating that pulmonary dysfunction after smoke exposure was progressive and became evident in the second 24 hrs. In group H, only one animal required ventilatory support, and in group LH, no animal required ventilatory support. The difference in the incidence of mechanical ventilatory support between S and LH was statistically significant, but that between groups S and H was not.

Figure 1 depicts the serial changes in $P(A\text{-}a)O_2$. $P(A\text{-}a)O_2$ increased progressively over 48 hrs in group S and H, whereas the increase was attenuated in group LH compared with group S during the latter half of the second 24 hrs. As a consequence, the change in $P(A\text{-}a)O_2$ over the 48-hr experimental period in group LH was significantly lower (by repeated-measures analysis of variance) than that of group S. In preliminary studies, treatment of smoke inhalation with lisofylline alone had no significant effect on $P(A\text{-}a)O_2$ at 48 hrs after smoke inhalation (48.5 ± 4.1 torr in the smoke-only group vs. 43.3 ± 2.6 torr in the smoke plus lisofylline group).

Table 2 shows the results of the MIGET analysis. The percentage of pulmonary shunt, i.e., the percentage of blood that perfuses essentially unventilated alveoli (Qs/Qt), calculated from the pulmonary capillary, venous, and arterial blood oxygen contents, was 37% in group S. Calculated Qs/Qt consists of shunt ($V_A/Q = 0$) and low V_A/Q area ($0 < V_A/Q < 0.1$). MIGET study demonstrated that $V_A/Q = 0$ area was 15% and low V_A/Q area was 22% in group S. Qs/Qt was significantly smaller in group LH than in group S. As shown in Table 2, the decrease in Qs/Qt was attributable to a decrease in the shunt compartment rather than the low V_A/Q area. There was no difference in any variable between groups H and S. As shown in Table 2, none of these variables in the preliminary study were significantly affected by lisofylline alone.

Table 3 summarizes the lung MDA concentrations and MPO activities, as well as

Table 1. Number of animals that required mechanical ventilation because of hypoxemia and hypercapnia

Group	12 Hrs	24 Hrs	36 Hrs	48 Hrs
S (n = 6)	0	0	1	4
H (n = 6)	0	0	0	1
LH (n = 6)	0	0	0	0[a]

S, group receiving nebulized saline; H, group receiving nebulized heparin; LH, group receiving heparin and lisofylline.

[a] $p < .05$ vs. group S.

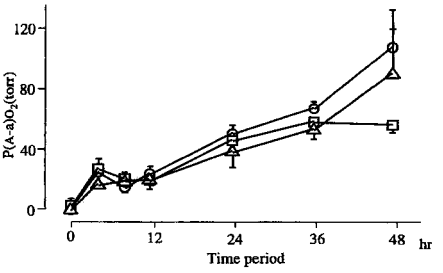

Figure 1. Serial alveolar-arterial oxygen tension gradient ($P(A\text{-}a)O_2$) after smoke exposure. The increase in $P(A\text{-}a)O_2$ was significantly attenuated in group LH at 48 hrs compared with group S ($p < .05$ vs. group S by analysis of variance with repeated measures). *Circle*, group S; *triangle*, group H; *square*, group LH. Data are expressed as mean ± SEM for six animals per group.

changes in the W/D in the lung. Lung MDA, an index of lipid peroxidation, was significantly less in group LH compared with group S. In preliminary studies, MDA was also significantly lower in L than S (Table 3). MPO activity, an index of neutrophil sequestration in the lung, was lower in group LH than in group S, but the difference did not reach significance. Similar observations were seen in the preliminary studies with L treatment alone. Despite these differences, W/D in group LH was similar to group S. That in group L in the preliminary study was also similar to group S. The W/D in group H tended to be less than in groups S and LH, but it did not differ significantly from either group.

Figure 2 depicts the change of serum total protein. Although a significant decrease was observed in all groups, the decrease in group LH was the greatest and the serial change was significantly different compared with group S. Table 4 summarizes volume of infusion, urine output, and water balance over the 48-hr experimental period in the three groups. Urine output was significantly less in LH than in S, resulting in water balance being higher in LH than in S. Further review reveals that the positive fluid bal-

Table 2. Results of multiple inert gas elimination technique study 48 hrs after smoke exposure

				Preliminary Studies	
	S	H	LH	S	L
Qs/Qt	36.6 ± 4.6	27.2 ± 9.4	20.8 ± 4.9[a]	28.6 ± 3.7	26.2 ± 3.1
$V_A/Q = 0$ area (% Q)	14.6 ± 5.2	10.0 ± 5.7	2.6 ± 2.6	6.3 ± 4.5	4.6 ± 2.6
$0 < V_A/Q < 0.1$ area (% Q)	22.0 ± 6.1	17.1 ± 5.5	18.2 ± 5.6	22.3 ± 6.5	21.6 ± 2.2

Qs/Qt, percentage of shunt calculated by CcO_2, CvO_2 and arterial oxygen content; $V_A/Q = 0$ area (% Q), percentage of blood flow to shunt; $0 < V_A/Q < 0.1$ area (% Q), percentage of blood flow to low V_A/Q area. S, group receiving nebulized saline; H, group receiving nebulized heparin; LH, group receiving heparin and lisofylline.

[a] $p < .05$ vs. S. Data expressed as mean ± SEM for six animals per group.

Table 3. Lung MDA, MPO, and W/D

				Preliminary Studies	
	S	H	LH	S	L
MDA	0.56 ± 0.09	0.43 ± 0.05	0.33 ± 0.06[a]	0.90 ± 0.05	0.70 ± 0.05[a]
MPO	0.21 ± 0.02	0.20 ± 0.02	0.18 ± 0.01[b]	0.16 ± 0.02	0.13 ± 0.009
W/D	6.6 ± 0.2	5.7 ± 0.3	6.7 ± 0.3	6.1 ± 0.3	6.2 ± 0.3

MDA, malondialdehyde (nmol/mg protein); MPO, myeloperoxidase (units/mg protein); W/D, wet to dry weight ratio; S, group receiving nebulized saline; H, group receiving nebulized heparin; LH, group receiving heparin and lisofylline.

[a] $p < .05$ vs. S; [b] $p = .14$ vs. S. Data expressed as mean ± SEM for six animals per group.

ance observed in the LH group could be accounted for by lower urine output in the first 24 hrs and higher fluid intake in the last 24 hrs relative to the other groups (Table 4). In the preliminary studies, 48-hr fluid balance was also higher in group L than S (26.4 ± 15.7 $mL \cdot kg^{-1} \cdot 48\ hrs^{-1}$ vs. 11.6 ± 11.1 $mL \cdot kg^{-1} \cdot 48\ hrs^{-1}$), although there were no significant differences in fluid intake or urine output over the 48-hr period (data not shown).

Tables 5 and 6 show the other cardiopulmonary variables after smoke exposure. Progressive hypoxemia that worsened after 24 hrs was seen in all groups. Because animals were ventilated if necessary, no statistical differences in Pa_{O_2} were observed among the groups. The increase in Pa_{CO_2} observed in group S was significantly attenuated in group LH. Although the mean values in group H were almost equal to those in group LH, there was no difference between groups S and H because of the greater variability of the data. Pulmonary vascular resistance index increased during the second 24 hrs in all groups. This increase was significantly attenuated in group H compared with both groups S and LH, but that in group LH was not attenuated. There were no significant differences among the groups in mean pulmonary artery pressure, cardiac index, pulmonary capillary occlusion pressure, mean arterial pressure, and total peripheral resistance index (Tables 5 and 6). No significant differences in hemodynamic variables were observed in the preliminary studies with lisofylline treatment alone (data not shown).

Table 7 shows the results of the histologic evaluation. Tracheobronchoepithelial injury was extensive in all groups, and there were no statistically significant differences among the groups. Lung parenchymal injury was less than observed in the tracheobronchial areas, but again there were no statistically significant differences among the three groups.

DISCUSSION

Activated leukocytes have been thought to play an important role in the development of pulmonary dysfunction after smoke inhalation. Consequently, many studies have focused on leukocyte modulation as a potential therapeutic target (6, 12, 18–20). However, those treatments alone may not be sufficient for severe smoke injury because they do not address the cast formation that typically narrows or occludes the airways during the second 24 hrs after injury (21). These findings support the hypothesis that the pathophysiology of smoke inhalation injury is complex, involving injury produced by leukocytes as well as

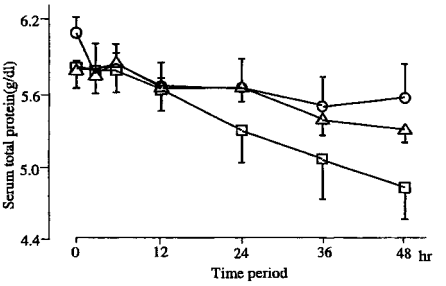

Figure 2. Serial serum total protein concentrations after smoke exposure. The decrease in group LH was the greatest and the serial change was significantly different compared with group S ($p < .05$ vs. group S by analysis of variance with repeated measures). *Circle*, group S; *triangle*, group H; *square*, group LH. Data are expressed as mean ± SEM for six animals per group. $p < .05$ vs. group S by repeated-measures analysis of variance, but p value at any point was not <.05 by *post hoc* Scheffé's test.

direct airway cytotoxity by components of the smoke.

A previous study at our institute suggested that pentoxifylline reduced inflammation and improved pulmonary function in a model of mild smoke inhalation injury in sheep (6). Because recent evidence suggested that lisofylline had significantly greater anti-inflammatory activity than pentoxifylline (7–10), we initiated a preliminary study to evaluate the efficacy of lisofylline in a sheep model of severe smoke inhalation injury. Although the results of that study showed no significant benefit when used alone, the data were encouraging enough to warrant investigating lisofylline, perhaps as an adjunctive treatment.

In the present study we chose to investigate the combined effects of lisofylline and heparin. The main action of heparin is an anticoagulant activity that may reduce cast formation by potentiating the antithrombin III-mediated inactivation of thrombin. Heparin also may act as a free radical scavenger (22–25). In the present study, we used nebulization as the route of heparin administration instead of intravenous infusion, because nebulization can deliver the drug to the injured airway in high concentration with little systemic effect. In our study, treatment with heparin alone reduced the increase in pulmonary vascular resistance index that occurred after 24 hrs but did not otherwise significantly affect pulmonary dysfunction. Anti-inflammatory effects of heparin were not evident in the current study because neither MDA nor MPO activities in the lung were lower in the heparin group than in the untreated smoke-

exposed animals. Moreover, in group H, Qs/Qt was not significantly less than in group S, suggesting that the effects of heparin alone were not sufficient to maintain the normal relationship between ventilation and blood flow and to prevent the pulmonary dysfunction caused by severe smoke injury.

Traber and coworkers (26, 27) used nebulized dimethylsulfoxide in addition to heparin to reduce free radical-induced injury and reported that combined treatment ameliorated the change in lung lymph flow and improved survival. In the present study, we administered lisofylline intravenously to attenuate the activation of circulating leukocytes after smoke exposure and reduce their migration into the injured sites (28). Lisofylline, although structurally similar to pentoxifylline, has a unique potential to suppress signaling by interleukin-12, a major inducer of interferon-γ and interleukin-2 (9). Lisofylline has been reported to reduce lung injury after hemorrhage, as well as after the administration of cytokines or *Pseudomonas aeruginosa* (29–32).

Combined treatment with systemic lisofylline and nebulized heparin in the present study appeared to improve pulmonary function compared with untreated smoke exposed sheep. It eliminated the progressive increase in $P(A\text{-}a)O_2$ between 36 and 48 hrs after smoke-induced injury and decreased the number of animals that required mechanical ventilatory support when compared with the S group, an effect not seen with lisofylline alone. Combined treatment decreased the Qs/Qt mainly because of a decrease in true shunt, a finding consistent with reduction of airway occlusion by prevention of cast formation and airway edema. The beneficial effects of the combined treatment also were associated with a significant reduction in lung MDA, supporting observations by us and others (33, 34) that free radical activity plays an important role in the pulmonary changes induced by smoke inhalation injury. Thus, data from the present and preliminary study support the "antioxidant" activity of lisofylline.

In the current study, there were no significant differences in lung MPO activity, an index of neutrophil sequestration, among the groups, although the mean value was lowest in the lisofylline-heparin group and was also lower in preliminary studies with lisofylline. Combined treatment appears to have improved lung function (matching of ventilation and perfusion) without decreasing leukocyte sequestration in the lung. However, because lung MPO activity was only determined at 48 hrs after smoke exposure, it is possible that leukocyte sequestration into lung early after smoke inhalation may have been reduced, but this hypothesis requires additional study. Also, because lisofylline alone in our preliminary study did not significantly attenuate pulmonary dysfunction despite the inhibition of leukocyte activation, heparin appears to be an important component of the combined treatment.

Table 4. Volume of infusion, urine output, and water balance

Group	mL/kg/12 hrs				Total (mL/kg/48 hrs)
	12	24	36	48	
Fluid intake, mL/kg					
S	24 ± 0	25.2 ± 0.21	28.8 ± 1.3	31.6 ± 1.2	109.6 ± 5.5
H	24 ± 0	24.8 ± 0.2	24.4 ± 0.1	32 ± 0.9	105.2 ± 2.8
LH	24 ± 0	28.8 ± 0.6	33.2 ± 2.0	38.0 ± 2.3	124.0 ± 12.1
Urine output, mL/kg					
S	32.9 ± 3.4	25.2 ± 2.1	27.2 ± 2.6	25.6 ± 2.4	117.2 ± 14.0
H	34.8 ± 1.5	27.2 ± 2.7	27.2 ± 2.7	15.2 ± 1.5	104.4 ± 16.2
LH	21.6 ± 4.8[a]	14.0 ± 1.0[a]	22.4 ± 2.0	21.6 ± 1.3	79.6 ± 5.1
Water balance, mL/kg					
S	−15.2 ± 3.5	0 ± 2.2	1.6 ± 3.3	6.0 ± 2.5	−7.6 ± 18.0
H	−10.8 ± 1.5	−2.4 ± 2.2	−2.8 ± 2.8	16.8 ± 2.3	0.8 ± 18.5
LH	2.4 ± 1.8[a]	14.8 ± 1.8[a]	10.8 ± 3.7	16.4 ± 2.2	44.4 ± 15.4

S, group receiving nebulized saline; H, group receiving nebulized heparin; HL, group receiving heparin and lisofylline.

[a] $p = .033$ vs. S. Data expressed as mean ± SEM for six animals per group.

Table 5. Cardiopulmonary indexes after smoke inhalation injury

Group	Baseline	4 Hrs	8 Hrs	12 Hrs	24 Hrs	36 Hrs	48 Hrs
Pa_{O_2}, torr[a]							
S	120 ± 6	95 ± 6	106 ± 2	96 ± 5	71 ± 7	55 ± 10	63 ± 5
H	121 ± 2	107 ± 2	103 ± 5	102 ± 7	86 ± 11	62 ± 8	60 ± 3
LH	117 ± 3	90 ± 7	100 ± 3	101 ± 3	75 ± 6	58 ± 3	59 ± 4
Pa_{CO_2}, torr							
S	36 ± 0.4	37 ± 1.3	35 ± 1.6	36 ± 1.1	35 ± 1.4	43 ± 4.5	61 ± 10.3
H	36 ± 0.7	37 ± 1.1	35 ± 1.2	35 ± 1.4	35 ± 1.4	38 ± 1.6	41 ± 2.6
LH[b]	36 ± 1.2	33 ± 1.5	34 ± 0.8	35 ± 1.2	33 ± 1.6	41 ± 4.7	41 ± 4.8
MPAP, mm Hg[a]							
S	16 ± 0.3	16 ± 1.0	15 ± 0.9	15 ± 0.8	17 ± 1.3	22 ± 2.2	25 ± 2.5[c]
H	15 ± 0.2	15 ± 0.8	15 ± 1.1	14 ± 0.3	15 ± 0.8	19 ± 0.9	21 ± 1.3[c]
LH	15 ± 0.6	15 ± 0.4	15 ± 0.5	16 ± 0.8	17 ± 1.2	21 ± 1.0	22 ± 1.0[c]
CI, $L \cdot min^{-1} \cdot m^2$							
S	7.5 ± 0.7	9.6 ± 1.6	7.9 ± 0.8	7.9 ± 1.3	6.8 ± 0.6	8.0 ± 1.3	6.9 ± 1.1
H	7.9 ± 0.8	9.0 ± 0.6	7.8 ± 0.6	7.8 ± 1.3	6.8 ± 0.6	8.1 ± 1.1	8.6 ± 1.0
LH	6.2 ± 0.5	7.5 ± 0.5	7.3 ± 0.7	7.5 ± 0.9	5.5 ± 0.4	6.0 ± 0.4	6.7 ± 0.4

S, group receiving nebulized saline; H, group receiving nebulized heparin; HL, group receiving heparin and lisofylline; MPAP, mean pulmonary artery pressure; CI, cardiac index.

[a] Significant time effect ($p < .05$) by analysis of variance with repeated measures; [b] serial change pattern significantly different compared with S ($p < .05$ by analysis of variance with repeated measures); [c] $p < .05$ from corresponding baseline value. Values are mean ± SEM for six animals per group.

Table 6. Cardiopulmonary indexes after smoke inhalation injury times

Group	Baseline	4 Hrs	8 Hrs	12 Hrs	24 Hrs	36 Hrs	48 Hrs
PCOP, mm Hg							
S	7 ± 0.4	6 ± 0.5	6 ± 1.0	6 ± 0.7	7 ± 1.1	8 ± 1.3	9 ± 1.0
H	6 ± 0.3	6 ± 0.4	6 ± 0.8	6 ± 0.2	7 ± 0.9	7 ± 1.1	8 ± 0.3
LH	6 ± 0.5	6 ± 1.0	7 ± 0.5	6 ± 0.6	7 ± 1.0	7 ± 0.7	7 ± 1.0
PVRI, dyne·sec/cm^5/m^2							
S	97 ± 13	85 ± 12	93 ± 13	99 ± 11	128 ± 15	149 ± 10	196 ± 22
H[a]	93 ± 9	83 ± 6	87 ± 6	92 ± 10	93 ± 5[c]	121 ± 10	123 ± 17[c]
LH[b]	110 ± 7	101 ± 12	95 ± 9	110 ± 11	154 ± 24[d]	185 ± 21[d]	179 ± 22
MAP, mm Hg							
S	96 ± 4	103 ± 7	98 ± 4	95 ± 6	101 ± 5	104 ± 8	118 ± 8
H	99 ± 2	100 ± 2	100 ± 2	95 ± 2	93 ± 3	96 ± 4	106 ± 7
LH	92 ± 2	101 ± 4	95 ± 2	94 ± 2	92 ± 2	93 ± 2	99 ± 5
TPRI, dyne·sec/cm^5/m^2							
S	1038 ± 76	925 ± 114	1014 ± 101	1021 ± 121	1213 ± 138	1206 ± 264	1509 ± 342
H	1031 ± 103	891 ± 63	1037 ± 79	1055 ± 115	1113 ± 105	1009 ± 143	1030 ± 148
LH	1161 ± 56	1093 ± 113	1069 ± 123	1035 ± 138	1313 ± 114	1196 ± 84	1134 ± 99

PCOP, pulmonary capillary occlusion pressure; S, group receiving nebulized saline; H, group receiving nebulized heparin; LH, group receiving heparin and lisofylline; PVRI, pulmonary vascular resistance index; MAP, mean arterial pressure; TPRI, total peripheral resistance index.

[a]Serial change pattern significantly different compared with S; [b]serial change pattern was significantly different compared with H ($p < .05$, by analysis of variance with repeated measures); [c]$p < .05$ vs. S; [d]$p < .05$ vs. H. Values are mean ± SEM for six animals per group.

Table 7. Histology damage score in airways and lung

	S	H	LH
Midtrachea	3.3 ± 0.5	3.3 ± 0.2	3.2 ± 0.2
Distal trachea	2.7 ± 0.6	3.3 ± 0.5	3.7 ± 0.2
Proximal bronchus	3.7 ± 0.2	3.7 ± 0.2	3.7 ± 0.2
Distal bronchus	3.8 ± 0.2	3.8 ± 0.2	3.7 ± 0.2
Lung apical lobe	2.2 ± 0.3	1.5 ± 0.3	1.8 ± 0.5

S, group receiving nebulized saline; H, group receiving nebulized heparin; LH, group receiving heparin and lisofylline. Data expressed as mean ± SEM from six animals per group.

Despite what appears to be beneficial effects of the combined treatment, some adverse effects also were observed, such as the greater fluid requirement to maintain urine output and the greater decrease in serum total protein concentration. These changes are consistent with lisofylline-induced water retention, but this has not been reported previously. More likely, these effects may be related to the prevention of cast formation, although such a conclusion seems paradoxic. The prevention of cast formation would maintain patency of the airways but may have resulted in a persistent exudation of secretions from the damaged airways. Cox et al. (4) also reported that intravenous administration of heparin increased tracheal fluid output. This fluid was a yellow, proteinaceous material that easily could be suctioned out of the airways. It is possible that in the present study, persistent proteinaceous exudation resulted in fluid loss, leading to the greater fluid requirement and greater decrease in serum protein observed in the LH than S group. Such a persistent serous exudation also could have contributed to the increase in pulmonary vascular resistance index if, for example, the fluid from the airways gravitated into the alveoli. Thus, the lung may have fallen into a situation of near-drowning where reflex pulmonary vasoconstriction can occur (35), leading to the observed increase in pulmonary vascular resistance index in the LH group. These secretions also could explain why there were no significant differences in W/D between the LH and S groups. If frequent suctioning of the airway secretions had been performed, perhaps we may have observed a lower W/D in the combined treatment group that would have been consistent with the lower MDA concentrations observed in this group compared with S.

An additional question is whether such increased secretions would result in greater shunt in the LH than S group, rather than the opposite effect observed. It is suggested that the shunt results from both occlusion of the airways by casts and alveolar flooding from secretions gravitating into alveoli. Because the degree of shunt would be expected to be greater if airway occlusion occurs at the level of the large bronchi compared with occlusion in the tertiary bronchi or alveoli, the mechanism by which the shunt fraction is attenuated by LH treatment (fluid in tertiary bronchi vs. occlusion of large bronchi by cast formation) may help explain the present observations and requires additional investigation.

As mentioned, there were no significant differences in airway or lung morphology injury score 48 hrs after smoke inhalation among the groups. The CO-Hb concentrations in the present study suggest a severe injury that was similar among the groups and consistent with other studies of smoke inhalation in sheep (17, 36, 37). The treatments in the present study would not be expected to affect the direct cytotoxic effects of smoke but could ameliorate the secondary injury resulting from smoke exposure. Unfortunately, the histologic grading used in this and other studies would not identify inhibition of cast formation or quantitate reduction in airway edema, which may be the mechanisms by which the combined treatment exerts its beneficial effects to improve pulmonary function. Further studies are needed to confirm our speculations.

The results of the present study suggest that the combined treatment evaluated would necessitate modification of current resuscitation regimens and would be difficult to adopt immediately into clinical practice. Frequent suctioning of airway secretions, administration of colloid containing fluid, and/or a different dosage of the two drugs may be required to optimize treatment of smoke inhalation injury in such patients, but this will require additional study.

Combined treatment with nebulized heparin and systemic lisofylline had beneficial effects on pulmonary function in association with a decrease in blood flow to poorly ventilated areas and less lipid peroxidation.

In summary, treatment of smoke inhalation injury with nebulized heparin alone in our model did not significantly attenuate pulmonary dysfunction. Combined treatment with nebulized heparin and systemic lisofylline had beneficial effects on pulmonary function as assessed by a decrease in the true shunt compartment, a lesser increase in $Paco_2$, reduced need for mechanical ventilation and oxygen supplementation, and reduction in lung MDA concentrations. On the other hand, the combined treatment was associated with a greater decrease in serum total protein concentrations and a greater fluid requirement compared with untreated smoke-exposed sheep. Because clinical trials with lisofylline alone to treat acute lung injury recently have been halted because of lack of a positive trend toward improved survival (38), combination treatment may be the best strategy. Additional research is necessary to assess the clinical applicability of this regimen.

ACKNOWLEDGMENTS

We are grateful to Rodney Koyle for technical support.

REFERENCES

1. Navar PD, Saffle JR, Warden GD: Effect of inhalation injury on fluid resuscitation requirements after thermal injury. *Am J Surg* 1985; 150:716–720
2. Demling R, Lalonde C, Youn YK, et al: Effect of graded increases in smoke inhalation injury on the early systemic response to a body burn. *Crit Care Med* 1995; 23:171–178
3. Herndon DN, Thompson PB, Traber DL: Pulmonary injury in burned patients. *Crit Care Clin* 1985; 1:79–96
4. Cox CS, Zwischenberger JB, Traber DL, et al: Heparin improves oxygenation and minimizes barotrauma after severe smoke inhalation in an ovine model. *Surg Gynecol Obstet* 1993; 176:339–349
5. Schenarts PJ, Traber LD, Bone HG, et al: Effects of porcine heparin on oxygenation and pulmonary transvascular fluid flux after smoke inhalation in sheep. *Crit Care Med* 1996; 24(Suppl):A89
6. Ogura H, Cioffi WG, Okerberg CV, et al: The effects of pentoxifylline on pulmonary function following smoke inhalation. *J Surg Res* 1994; 56:242–250
7. Lillibridge JA, Kalhorn TF, Slattery JT: Metabolism of lisofylline and pentoxifylline in human liver microsomes and cytosol. *Drug Metab Disp* 1996; 24:1174–1179
8. Rice GC, Rosen J, Weeks R, et al: CT-1501R selectively inhibits induced inflammatory monokines in human whole blood ex vivo. *Shock* 1994; 4:254–266
9. Waxman K, Daughters K, Aswani S, et al: Lisofylline decreases white cell adhesiveness and improves survival after experimental hemorrhagic shock. *Crit Care Med* 1996; 24:1724–1728
10. Bright JJ, Du C, Coon M, et al: Prevention of experimental allergic encephalomyelitis via inhibition of IL-12 signaling and IL-12 mediated Th1 differentiation: An effect of the novel anti-inflammatory drug lisofylline. *J Immunol* 1998; 161:7015–7022
11. Wattanasirichaigoon S, Menconi MJ, et al: Lisofylline ameliorates intestinal mucosal barrier dysfunction caused by ischemia and ischemia/reperfusion. *Shock* 1999; 11:269–275
12. Tasaki O, Mozingo DW, Ishihara S, et al: Effects of Sulfo Lewis C on smoke inhalation injury in an ovine model. *Crit Care Med* 1998; 26:1238–1243
13. Rodriquez-Roisin R, Wagner PD: Clinical relevance of ventilation-perfusion inequality determined by inert gas elimination. *Eur Respir J* 1990; 3:469–482
14. Ogura H, Cioffi WG, Jordan BS, et al: The effects of inhaled nitric oxide on smoke inhalation injury in an ovine model. *J Trauma* 1994; 37:294–302
15. Naito C, Kawamura M, Yamamoto Y: Lipid peroxides as the initiating factor of atherosclerosis. *Ann N Y Acad Sci* 1993; 676:27–45
16. Trush MA, Egner PA, Kensler TW: Myeloperoxidase as a biomarker of skin irritation and inflammation. *Food Chem Toxicicol* 1994; 32:143–147
17. Tasaki O, Goodwin CW, Saitoh D, et al: Effects of burn on inhalation injury. *J Trauma* 1997; 43:603–607
18. Basadre JO, Sugi K, Traber DL, et al: The effect of leukocyte depletion on smoke inhalation injury in sheep. *Surgery* 1988; 104:208–215
19. Niehaus GD, Kimura R, Traber LD, et al: Administration of synthetic antiprotease reduces smoke-induced lung injury. *J Appl Physiol* 1990; 69:694–699
20. Nguyen TT, Cox CS, Herndon DN, et al: Effects of manganese superoxide dismutase on lung fluid balance after smoke inhalation. *J Appl Physiol* 1995; 78:2161–2168
21. Traber DL, Herndon DN: Pathophysiology of smoke inhalation. *In:* Respiratory injury: Smoke inhalation and burns. Haponik EF, Munster AM (Eds). New York, McGraw-Hill, 1990, pp 61–71
22. Goode HF, Richardson N, Myers DS, et al: The effect of anticoagulant choice on apparent total antioxidant capacity using three different methods. *Ann Clin Biochem* 1995; 32:413–416
23. Albertini R, Rindi S, Passi A, et al: The effect of heparin on Cu^{2+}-mediated oxidation of human low-density lipoproteins. *FEBS Lett* 1995; 377:240–242
24. Albertini R, Rindi S, Passi A, et al: Heparin protection against Fe2+- and Cu2+-mediated oxidation of liposome. *FEBS Lett* 1996; 383:155–158
25. Grant D, Long WF, Mackintosh G, et al: The antioxidant activity of heparins. *Biochem Soc Trans* 1996; 24:194S
26. Brown M, Desai M, Traber LD, et al: Dimethylsulfoxide with heparin in the treatment of smoke inhalation injury. *J Burn Care Rehabil* 1988; 9:22–25
27. Kimura R, Traber LD, Herndon DN, et al: Treatment of smoke-induced pulmonary injury with nebulized dimethylsulfoxide. *Circ Shock* 1988; 25:333–341
28. Traber DL, Linares HA, Herndon DN: The pathophysiology of inhalation injury—A review. *Burns* 1988; 14:357–364
29. Abraham E, Bursten S, Shenkar R, et al: Phosphatidic acid signaling mediates lung cytokine expression and lung inflammatory injury after hemorrhage in mice. *J Exp Med* 1995; 181:569–575
30. Hasegawa N, Oka Y, Nakayama M, et al: The effects of post-treatment with lisofylline, a phosphatidic acid generation inhibitor, on sepsis-induced acute lung injury in pigs. *Am J Respir Crit Care Med* 1997; 155:928–936
31. Hybertson BM, Bursten SL, Leff JA, et al: Lisofylline prevents leak, but not neutrophil accumulation, in lungs of rats given IL-1 intratracheally. *J Appl Physiol* 1997; 82:226–232
32. Guidot DM, Bursten SL, Rice GC, et al: Modulating phosphatidic acid metabolism decreases oxidative injury in rat lungs. *Am J Physiol* 1997; 273:L957–L966
33. Dubick MA, Jordan BS, Carden SC, et al: Antioxidant status of bronchoalveolar lavage fluid and lungs from rats subjected to burn and/or smoke inhalation. *Toxicologist* 1998; 42:348
34. Lalonde C, Picard L, Campbell C, et al: Lung and systemic oxidant and antioxidant activity after graded smoke exposure in the rat. *Circ Shock* 1994; 42:7–13
35. Levin DL, Morriss FC, Toro LO, et al: Drowning and near-drowning. *Pediatr Clin North Am* 1993; 40:321–336
36. Shimazu T, Yukioka T, Hubbard GB, et al: A dose-response model of smoke inhalation injury. Severity-related alterations in cardiopulmonary function. *Ann Surg* 1987; 206:89–98
37. Ahn SY, Sugi K, Talke P, et al: Effects of allopurinol on smoke inhalation in the ovine model. *J Appl Physiol* 1990; 68:228–234
38. Anonymous. Lisofylline. *Drug Future* 2000; 25:522–523